Docket No. 1676.002US1 Client Ref. No. CRF-D-2484A

CLEAN VERSION OF AMENDED SPECIFICATION PARAGRAPHS

ANTIGEN FOR DEVELOPING NEUTRALIZING ANTIBODIES TO HUMAN IMMUNODEFICIENCY VIRUS
Applicant: Min Lu et al.
Serial No.: 09/877,606

Clean Version of the Paragraph Beginning at Line 10, Page 5:

B1 Figure 2B schematically depicts a chimera of gp41 (residues 536 to 666) and the GCN4-pII peptide (*14*). The amino acid sequence of the COOH-terminal extension of gp41 is shown in an expanded view (SEQ ID NO:1). Residues in the *a* and *d* positions of gp41 residues, and GCN4-pII in heptad register, are indicated. A continuous helix is assumed between the gp41 coiled coil and GCN4-pII. The locations of serine substitutions for Ile^{573} and Leu^{576} in the NH_2-terminal heptad-repeat region are indicated by arrows.

Clean Version of the Paragraph Beginning at Line 19, Page 5:

B2 Figure 3A shows the helical wheel projection of C45-pII (SEQ ID NO:2). View is from the NH_2 terminus. The amino acid sequence of C45 (residues 624 to 668) is shown in red. The residues in black are from GCN4-pII. The residue in blue corresponds to a conservative leucine substitution for Met^{629}, made to facilitate protein production.

Clean Version of the Paragraph Beginning at Line 3, Page 6:

B3 Figure 4A shows the amino acid sequence of C52 with the *a* and *d* positions of the heptad repeat indicated above the sequence (SEQ ID NO:3). The sequence of the C45 peptide is also indicated.

Clean Version of the Paragraph Beginning at Line 8, Page 7:

B4 Figure 7A illustrates the chimera of the C52 peptide (residues 624 to 675) and an isoleucine-zipper trimer (GCN4-pII) (SEQ ID NO:4). Met629 in the C52-pII molecule was mutated to leucine to facilitate protein production. The location of the C45 segment (residues 624 to 668) is indicated. The gp41 residues are numbered according to their position in gp160 of the HXB2 HIV-1 strain.

Clean Version of the Paragraph Beginning at Line 13, Page 7:

B5 Figure 7B is a helical wheel representation of C52-pII (SEQ ID NO:4). The sequence of C52 is shown in red, while the residues in black are from GCN4-pII. A continuous helix is assumed between the C52 coiled coil and GCN4-pII. View is from the NH2 terminus.

Clean Version of the Paragraph Beginning at Line 24, Page 7:

B6 Figure 8A shows the frequency of occurrence of amino acids at positions 669 to 675 of gp160 (SEQ ID NO:5 and SEQ ID NO:8). The sequences of 213 fully sequenced M group HIV-1 strains (HIV Sequence Database [1998/1999 alignments], Los Alamos National Laboratory, http:hiv-web.lanl.gov) were analyzed. The amino acids occurring at positions 669 to 675 are shown with the number of times they occur at these positions. Periods indicate stop codons.

Clean Version of the Paragraph Beginning at Line 14, Page 22:

B7 cont'd. The genes of gp41 (residues 536 to 666, numbered according to their position in gp160 the HXB2 HIV-1 strain) and GCN4-pII (*14*) were subcloned into the expression vector pTMHa [J. P. Staley and P. S. Kim, *Protein Science* **3**, 1822 (1994)] to produce plasmid pRgp41pII (Fig. 2B). Met^{629} in the pRgp41pII construct was mutated to leucine by single-stranded mutagenesis [T. A. Kunkel, J. D. Roberts, R. A. Zakour, *Methods Enzymol.* **154**, 367 (1987)]. An expression

vector (pC45pII) encoding the C45-pII peptide was derived from pRgp41pII by PCR amplification followed by subcloning into pTMHa. The *Kpn*I site in pC45pII was replaced by the coding sequence for the residues Ala-Ser. Plasmid pC45 was derived from pC45pII. Plasmid pC52 was derived from pC45pII by the insertion of the appropriate DNA sequences encoding the residues Leu-Trp-Asn-Trp-Phe-Asn-Ile (SEQ ID NO:7) in place of GCN4-pII. Plasmid pC29p was derived from pC45pII. Single letter abbreviations for amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

Clean Version of the Paragraph Beginning at Line 14, Page 26:

Correlation with genetic studies. In the trimer-of-hairpins structure of fusion-active gp41, the conserved Gln^{652} and Asn^{656} residues at the ***d*** and ***a*** positions of the outer helix respectively are packed into a hydrophobic groove on the surface of the NH_2-terminal coiled coil (*6*). Whereas the Asn^{656} to Leu mutation abolishes membrane fusion, the Gln^{652} to Leu mutation increases HIV-1 infectivity (*17*). To define the basis for these *in vivo* phenotypes, the role of Gln^{652} and Asn^{656} on coiled-coil interactions was investigated by characterizing variants of the C52 peptide: Q652L and N656L (SEQ ID NO:9). The Q652L peptide was derived form the C52 peptide (residues 624-675) by substituting a leucine for glutamine at position 652. The N652L peptide was also derived from the C52 peptide by substituting a leucine for asparagine at position 656. Both peptides were made by single stranded mutagenesis using the method described in Kunkel, T.A., et al., *Methods Enzymol.* 154, 367 (1987), and produced by bacterial expression. On the basis of CD measurements at 100 μM peptide concentration in PBS (pH 7.0) at 0°C, Q652L is ~70% helical and N656L (SEQ ID NO:9) is fully helical (Fig. 6A). Under these conditions, Q652L displays a broad thermal unfolding transitions and N656L melts cooperatively with a T_m of 43°C (Fig. 6B). The Q652L peptide sediments as a trimer at concentrations between 30 and 100 μM (Fig. 6C). The N656L peptide does not sediment as a

unique oligomeric species; its apparent molecular weight changes significantly with peptide concentration (Fig. 6C). The Gln^{652} to Leu substitution therefore essentially exerts no effects on formation of C52 trimers, while the Asn^{656} to Leu substitution imparts strong helical character at the expense of structural uniqueness.

Clean Version of the Paragraph Beginning at Line 4, Page 32:

Given the similarity in structure between C52-pII and C45-pII, and the identity in the gp41 portion between C52-pII and C52, it appears that a trimeric coiled-coil segment formed by the sequence LWNWFNI ((SEQ ID NO:7); amino acids 669 to 675 of gp41) is involved in raising neutralizing antibodies. Trp^{670} and Trp^{672} are completely conserved in 213 fully sequenced M group HIV-1 strains, while there is only a single conservative methionine substitution for Ile^{675}. In addition, Ile^{669} and Phe^{673} occur in 205 and 204 of the 213 sequences respectively, with only a single nonconservative change present at each position. Even two more variable positions (671 and 674) are occupied by single polar and negatively charged residues in more than 75 and 60% of the sequences respectively; all the remaining sequences at each position possess conservative changes. It appears therefore, that there is selective pressure on the *a* and *d* positions to maintain trimeric coiled-coil interactions, as well as pressure on the outside heptad positions to preserve particular types of amino acid character. These conserved sequence and structural elements likely underlie the ability of C52-pII to elicit broadly reactive neutralizing antibodies, thus making it an attractive candidate for vaccine efforts.

Clean Version of the Paragraph Beginning at Line 18, Page 32:

Immediately NH_2-terminal to the sequence LWNWFNI (SEQ ID NO:7) is an epitope (the linear sequence ELDKWA; SEQ ID NO:6) recognized by the human antibody 2F5 isolated from an HIV-1-infected donor (*S1*). Because the immunizing C52-pII peptide was able to abrogate the fusion inhibition activity of the neutralizing antibodies, whereas C52 and C45-pII (both

containing ELDKWA) were not (Figure 8), it is likely that the discontinuous neutralization determinant defined in this study is different from the continuous 2F5 epitope. Consistent with this notion is the observation that C85-pIIB serum and 2F5 represent different specificities in neutralizing seven primary clinical isolates tested (Table 1). Thus, the conserved, conformation-specific epitope in the C52-pII 'tailor-made' molecule appears to produce more effective antibody responses than a natural HIV-1 infection.